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Cilia from Abalone Larvae Contain a Receptor-Dependent G Protein Transduction System Similar to that in Mammals

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Abstract. Lysine and related diamino acids amplify (facilitate) the response to inducers of metamorphosis in larvae of the marine mollusk *Haliotis rufescens*. Previous studies showed that a cholera toxin-sensitive G protein transduces the lysine signal via a diacylglycerol-dependent pathway. We have isolated and partially purified larval cilia that may be involved in recognizing the facilitating chemical signals. These isolated cilia provide an open or porous membrane-associated sensory system that is uniquely tractable for *in vitro* analyses of chemosensory signal transduction. The cilia contain receptors that exhibit sodium-independent binding of the facilitating diamino acids. The binding strength for lysine and related diamino acids *in vitro* is correlated with the effectiveness of these ligands as facilitators *in vivo*. The cilia contain a cholera toxin-sensitive G protein functionally coupled to the lysine receptor. The receptor and the G protein reciprocally regulate one another, suggesting that the chemosensor may be a member of the rhodopsin-like, G protein-coupled transmembrane receptor superfamily. Previous analyses of mRNAs from the larval cilia revealed a sequence coding for a G protein with high homology to G_q from mammalian brain, and another sequence coding for a protein homologous to G_i/G_o. Similarities between this system, other chemosensory signal transduction pathways, and mechanisms of neuronal long-term potentiation are evident. Because the receptors and transducers controlling settlement and metamorphosis in *Haliotis* and other marine invertebrate larvae appear homologous to components controlling neuronal activity, cellular proliferation, and differentiation in mammals, characterization of the molecules controlling metamorphosis may help in the design of new regulators useful in medicine.

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Abbreviations: G protein, guanine nucleotide binding protein; GABA, γ-aminobutyric acid; GppNHp, guanosine 5′ (β,γ-imido) triphosphate; PKC, protein kinase C; ASW, artificial seawater; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DAPA, L-α,β-diaminopropionic acid; GDP-β-S, guanosine 5′-0-[β-thio] diphosphate; PLC, phospholipase C; Tris, tris-hydroxymethylaminomethane.

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Introduction

The molecular components and mechanisms controlling the settlement and metamorphosis of marine invertebrate larvae in response to chemical signals from the environment are strikingly similar, in some cases, to those that mediate responses to hormones, transmitters, and other signals regulating neuronal activity, behavior and cellular differentiation in mammals. These processes in the larvae, like their counterparts in mammals, also exhibit far greater complexity than first realized. We present here recent findings on one such system from a molluscan larva, and discuss these data in the context of recently discovered sequence homologies and other evidence for relatedness to mammalian receptor-transducer pathways in molluscan and coral larval systems.

Larvae of the marine gastropod mollusk *Haliotis rufescens* (red abalone) are induced to settle from the plankton and metamorphose in response to exogenous γ-aminobutyric acid (GABA)[1]-mimetic peptides found on the surfaces of specific algae; GABA and GABA analogs also induce this metamorphosis (Morse *et al.*, 1979, 1980; Morse and Morse, 1984; Morse, 1985, 1990). These morphogenic inducers are recognized by externally accessible chemosensory receptors that have been characterized by radioligand binding and competition studies (Trapido-Rosenthal and Morse, 1986a). The induction of meta-

morphosis of *Haliotis* larvae by low concentrations of these inducers can be facilitated, or amplified, by lysine and structurally related diamino acids (Trapido-Rosenthal and Morse, 1985). Experiments *in vivo* showed that transduction of the facilitating diamino acid signal is mediated by a regulatory pathway that is separate from the morphogenetic pathway (Trapido-Rosenthal and Morse, 1986a, b; Baxter and Morse, 1987).

From experiments conducted with *Haliotis* larvae *in vivo*, we concluded that a cholera toxin-sensitive G protein transduces the facilitating diamino acid signal, and that this G protein may control a phospholipase C-diacylglycerol-protein kinase C cascade (Baxter and Morse, 1987). We report here the purification of epithelial cilia from *Haliotis* larvae, and the finding that a chemosensory diamino acid (lysine) receptor is located on these isolated cilia. These cilia also contain a cholera toxin-sensitive G protein functionally coupled to the lysine receptor. These observations, and the recent discovery of mRNAs coding for G proteins in the isolated cilia (Wodicka and Morse, 1991), support the suggestion that some of these cilia may have a chemosensory function. The lysine receptor and G protein reciprocally regulate one another in the isolated cilia, in a manner similar to that observed for transmembrane receptor-controlled G proteins in mammalian systems. In conjunction with the recently reported high sequence homology between one of the ciliary G proteins and a G protein from mammalian brain (Wodicka and Morse, 1991), these results indicate that similar signal transduction pathways and components may be central to regulation in both larval metamorphosis and mammalian neuronal function and cell differentiation.

Materials and Methods

Larvae and materials

Haliotis rufescens larvae were produced and cultured at 15°C at a density of 10^5 larvae per liter, as described previously (Morse *et al.*, 1979, 1980). Under these conditions, larvae become competent for metamorphosis 7 days post-fertilization (Morse *et al.*, 1980). Forty-eight hours prior to use, competent larvae were transferred to fresh 5 μm filtered ultraviolet sterilized seawater containing 2 μg/ml rifampicin, at a density of 10^5 larvae/liter at 15°C. The radiochemicals [^{32}P]-NAD, [^{3}H]-L-lysine, [^{3}H]-L-alanine and [^{3}H]-L-leucine were purchased from DuPont/NEN. Electrophoresis reagents were purchased from Bio-Rad Laboratories. All other chemicals were purchased from Sigma. Artificial seawater media (see below) were aerated for 16 h at 15°C before use.

Cilia isolation

Abscission of cilia was induced by a modification of the calcium/ethanol procedure developed by Watson and Hopkins (1962). Prior to removal of cilia, 10^6 larvae at 7 days post-fertilization were incubated at 15°C in 18 l of a calcium-free artificial seawater (ASW) solution (420 m*M* NaCl, 9.0 m*M* KCl, 52 m*M* $MgSO_4$, 23 m*M* $MgCl_2$, 2.0 m*M* $NaHCO_3$, 9.0 m*M* Tris-HCl, and 2 μg/ml rifampicin; final pH = 7.8) for 24 h, and then transferred to normal ASW (Cavanaugh, 1956) containing rifampicin (2 μg/ml) for a further 24 h incubation at 15°C to allow equilibration to normal conditions. This treatment effectively removes the larval shell, thus exposing the epithelial cilia (located primarily on the head). Approximately 10^6 treated larvae then were placed in 135 ml ASW to which ethanol was added to a final concentration of 10% (v/v); this mixture was gently stirred for 1 min at room temperature. $CaCl_2$ was added to a final concentration of 10 m*M* over that in ASW, and the sample stirred for an additional 17 min at room temperature. After filtration through a 95 μm nylon mesh (Nitex brand) to remove deciliated larvae, the suspension of shed cilia was centrifuged at 1500 × *g* for 5 min at 4°C to precipitate debris; the cilia then were collected by centrifugation at 10,000 × *g* for 10 min at 4°C. The resulting cilia pellet was washed three times by resuspension in 30 ml buffer [20 m*M* Tris-HCl (pH 7.6), 10 m*M* $MgCl_2$, 1 m*M* EGTA] and centrifugation at 10,000 × *g* for 10 min at 4°C. The final washed pellet was resuspended in buffer (as specified below). Protein was measured spectrophotometrically (Bradford, 1976) after alkaline digestion.

Scanning electron microscopy

The cilia pellet was suspended in water and applied to poly-L-lysine-treated glass cover slips. After 30 min, the cover slips with affixed cilia were washed with water, subjected to critical-point drying, sputtercoated with gold, and examined with an ISI Model Alpha-9 scanning electron microscope.

Radioligand binding assays

Binding of (^{3}H)-L-lysine was analyzed as a function of concentration with minor modifications of the method previously described (Trapido-Rosenthal and Morse, 1986a). Cilia were apportioned at a final protein concentration of 50 μg/ml in 250 μl final volume of binding buffer (20 m*M* Tris-HCl pH 7.6, 10 m*M* $MgCl_2$), and incubated with radioactive ligand for 1 h at 2°C. Assays were terminated by filtration through 0.2 μm nitrocellulose membrane filters (Millipore HAWP; 25 mm diam.) that had been presoaked in buffer at 0°C for 20 min. Filters were rinsed rapidly with two successive 3-ml portions of ice-cold buffer and dissolved in scintillation fluid; radioactivity was determined by liquid scintillation. Scatchard analyses of binding data were performed with the non-linear curve fitting programs EBDA/LIGAND of

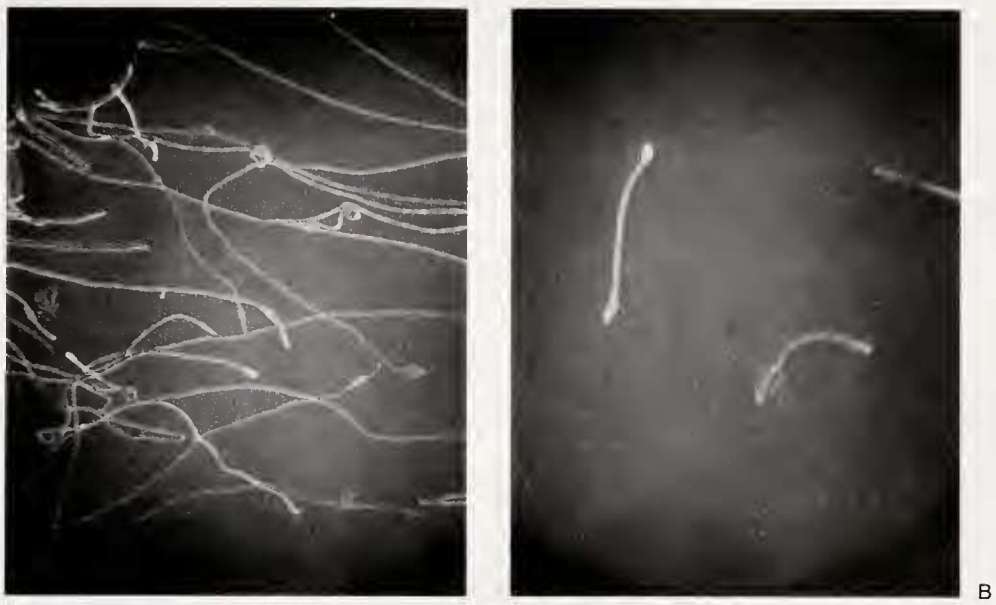

Figure 1. Scanning electron micrographs of the cilia purified from *Haliotis* larvae. (A) Scanning electron micrograph of cilia preparation (2400×). Contour lengths of the long propulsive cilia ≥ 5.0 μm. (B) Isolated view of smaller proposed sensory cilia (length *ca.* 0.5 μm) in the same preparation (4500×).

Rodbard and Munson (McPherson, 1985). Competition for binding by unlabelled L-lysine, L-ornithine, or L-α,β-diaminopropionic acid was analyzed similarly, with the same program.

ADP-ribosylation

Cholera toxin-catalyzed ADP-ribosylation for the labeling of G protein was performed as described by Pace and Lancet (1986), using cholera toxin preactivated by incubation with 20 m*M* dithiothreitol at room temperature for 15 min. Cilia (200 μg/ml protein) were incubated at room temperature in a buffer containing 20 m*M* Tris-HCl (pH 7.6), 30 m*M* thymidine, 1 m*M* ATP, 0.1 m*M* GTP, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 3 m*M* phosphoenolpyruvate, 5 units/ml pyruvate kinase, 0.05% Triton X-100, 1 m*M* dithiothreitol, 5 μ*M* [^{32}P]-NAD (10–20 Ci/mmol), and preactivated cholera toxin at a final concentration of 10 μg/ml in a final volume of 200 μl. The reaction was terminated after 30 min by adding an equal volume of 2× electrophoresis sample buffer (100 m*M* Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 100 m*M* dithiothreitol, and 0.004% bromophenol blue) and boiling for 20 min. ADP ribosylation of cilia for G protein activation was conducted under similar conditions without radioisotope (Pace and Lancet, 1986), using a 1-h incubation. Cilia then were centrifuged at 15,000 × *g* for 10 min at 4°C, resuspended in buffer, and assayed as described.

Gel electrophoresis and autoradiography

Proteins were separated by one-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) with a 10% separating gel. Molecular weight standards were included on each gel. Gels were dried and exposed to Kodak X-OMAT x-ray film with intensifying screens at −70°C. Bands were quantified by densitometric scanning of the autoradiogram with an LKB Ultra Scan XL.

Results

Isolation of larval cilia

Cilia were purified from *Haliotis rufescens* larvae as described above; the yield was about 0.8 g wet weight/10^6 larvae. The resulting preparation contained cilia recognizably derived from externally accessible structures, apparently including the propulsive swimming cilia from cells of the velum and sensory cilia from the cephalic apical tuft and other sensory structures (Bonar, 1978; Yool, 1985). The deciliated larvae remained alive and largely intact, indicating that the cilia obtained were removed from external epithelia. Scanning electron microscopy revealed a highly purified but heterogeneous population of cilia with no apparent contamination by other cellular material (Fig. 1). Two distinct classes are seen: elongated cilia (≥5 μm length), apparently the swimming cilia from the velum, and short cilia (*ca.* 0.5 μm long), each with an enlarged process at one end (Fig. 1B); these apparently include the paddle cilia of the apical tuft and other sensory tissues. [Although the distal enlargement is clearly an artifact of fixation, its distribution in any organism is limited to particular body regions (Short and Tamm, 1991). In

larval *Haliotis refescens* (cf. [illegible]ol, 1985)—as in marine turbellaria (Ehlers and Ehlers, 1978)—paddle cilia are found only on sensory cilia, reflecting, presumably, a unique specialization. SDS-gel electrophoresis confirmed the substantial purification of the larval cilia (Baxter, 1991); a dominant protein with molecular mass identical to that of α and β tubulin was found in the cilia preparation, whereas this was only a minor constituent of the proteins from the whole larvae.

Lysine receptors

The purified cilia exhibit specific and saturable binding of [^{3}H]-L-lysine that reaches equilibrium within 15 min at 2°C (Baxter, 1991). Scatchard analysis of the specific equilibrium binding (Fig. 2) formally resolves a high affinity binding site ($K_d \leq 0.1\ \mu M$) and a low affinity site ($K_d \geq 10\ \mu M$). The maximal binding (B_{max}) of lysine is on the order of 10 femtomoles/μg cilia protein at the high affinity site and *ca.* 100 femtomoles/μg protein at the low affinity site. Although exhibiting some variation from one preparation of cilia to another, these binding parameters proved highly reproducible within each preparation. The presence or absence of sodium had no significant effect on these parameters for lysine binding (Baxter, 1991).

The abilities of the unlabeled facilitating diamino acids L-α,β-diaminopropionic acid (DAPA), L-lysine and L-ornithine to compete for the specific binding of [^{3}H]-L-lysine to the cilia are correlated with the activities of these compounds as facilitators of larval metamorphosis (Fig. 3). The rank order of binding strength and biological effectiveness is DAPA > L-lysine > ornithine.

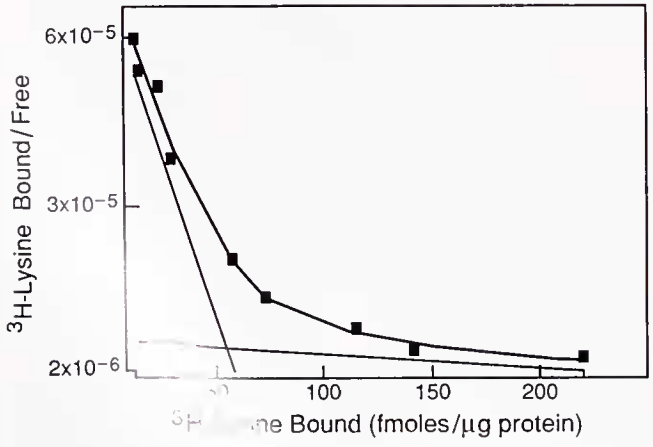

Figure 2. Binding [illegible] L-lysine to cilia purified from *Haliotis* larvae. Scatchard analysis of equilibrium specific binding data. Data were analyzed with the computer assisted non-linear curve fitting programs EBDA/LIGAND (McPherson, 1985); each point represents the mean of three determinations. Details as described in Materials and Methods.

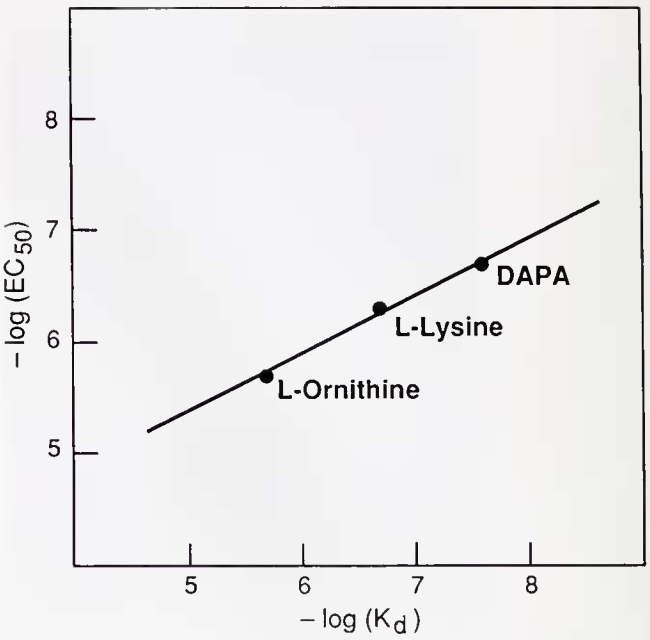

Figure 3. Comparison of the ciliary binding affinities of DAPA, L-lysine and L-ornithine to the relative biological effectiveness of these compounds as facilitators of the larval morphogenetic response. The apparent dissociation constants were determined by competition binding studies with [^{3}H]-L-lysine and analyses of data using the computer programs EBDA/LIGAND as described in Materials and Methods. The EC_{50} value is the concentration of diamino acid that produces 50% of the maximal amplification of the morphogenetic response induced by 0.1 μM GABA. This was determined using the biological assay of settlement and metamorphosis described in detail elsewhere (Trapido-Rosenthal and Morse, 1985).

Receptor coupled to G protein

We previously reported that cholera toxin and the nonhydrolyzable GTP analog 5′-guanylylimidodiphosphate (GppNHp), both activators of G proteins, facilitate the morphogenetic response of *Haliotis* larvae to GABA in a manner similar to that of the diamino acids, thus implicating G protein in this control (Baxter and Morse, 1987). Labeling with cholera toxin, which catalyzes ADP-ribosylation of susceptible G protein α subunits (Schleifer *et al.*, 1980), reveals this protein in the isolated cilia. A major polypeptide substrate for this reaction was found with an apparent molecular mass of 45 kDa (Baxter, 1991), in the range reported for the α subunits of cholera toxin-sensitive G proteins from a variety of sources (Schleifer *et al.*, 1980; Hurley *et al.*, 1984; Gilman, 1987). We show here that this ADP-ribosylation of the ciliary protein catalyzed by cholera toxin is stimulated as much as 10-fold by the nonhydrolyzable GTP analog, GppNHp, in a concentration-dependent manner (Fig. 4).

Direct activation of G protein in the cilia with GppNHp reciprocally affects the lysine receptors, increasing the K_d of the high affinity lysine receptor 17-fold, with little effect

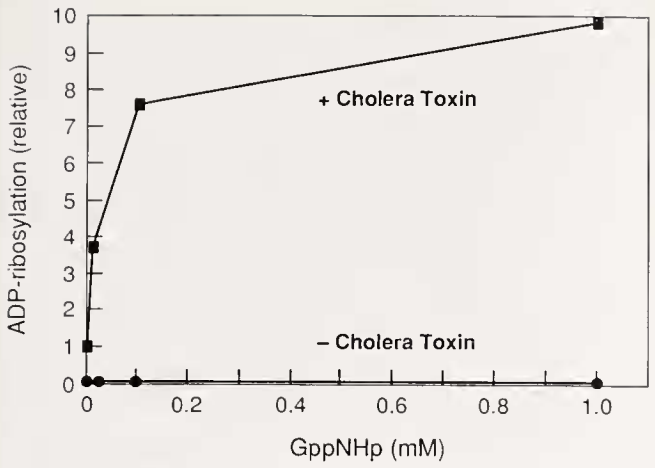

Figure 4. Stimulation of cholera toxin-dependent ADP-ribosylation by GppNHp. Isolated cilia were incubated with [^{32}P]-NAD in the presence and absence of 10 μg/ml cholera toxin and increasing concentrations of GppNHp. The treated cilia then were solubilized by boiling in SDS-PAGE sample buffer and proteins were separated on 10% SDS polyacrylamide gels followed by autoradiography. The values presented were determined from densitometric scans of the 45 kDa bands of the autoradiogram; relative ADP-ribosylation was normalized to ^{32}P-ADP-ribose incorporation in the absence of added GppNHp.

on total binding (Table I). Pretreatment of the cilia with cholera toxin produces a similar effect (14-fold increase in K_d; Table I). Thus we conclude that both GppNHp and cholera toxin, agents that facilitate the larval response *in vivo* (Baxter and Morse, 1987) and interact with a ciliary G protein *in vitro* (Fig. 4; *cf.* Baxter, 1991), reciprocally modulate the affinity of the ciliary lysine receptor *in vitro*.

Discussion

Facilitation and long-term potentiation

Lysine and its analogs facilitate the response of *Haliotis* larvae to low concentrations of morphogenetic stimuli, apparently increasing the sensitivity or output of the morphogenetic pathway without affecting the K_d or B_{max} of the receptors that bind the morphogenetic GABA analogs (Trapido-Rosenthal and Morse, 1985, 1986a, b; Morse, 1990). The receptors and signal transducers of the lysine-dependent regulatory or amplifier pathway prove to be distinct from those of the morphogenetic pathway (Baxter and Morse, 1987; Morse, 1990). In *Haliotis* larvae, the facilitation (amplification) of responsiveness to morphogenetic stimuli is long-lived, persisting for at least several days (Trapido-Rosenthal and Morse, 1986b). This facilitation is thus similar, both functionally and biochemically, to mechanisms of long-term potentiation and classical conditioning (learning) in other systems (*cf.* Greenberg *et al.*, 1987; Malenka *et al.*, 1987; Malinow *et al.*, 1989; Sweatt and Kandel, 1989; Nelson *et al.*, 1990). The possible adaptive significance of this system for the dual regulation of larval site-selection and metamorphosis by convergent chemosensory pathways may include the capacity for fine-tuning site selection behavior in response to chemical characteristics of the environment, with enhanced selection of sites for metamorphosis (irreversible commitment to a sessile habit) in potentially favorable areas (Trapido-Rosenthal and Morse, 1986b; Baxter and Morse, 1987; Morse, 1990).

Functional coupling of ciliary receptor and G protein

Evidence obtained *in vivo* demonstrated that the regulatory pathway is controlled by chemosensory receptors specific for lysine and lysine analogs, and that the lysine binding signal is transduced by a receptor-regulated G protein-dependent cascade (Baxter and Morse, 1987). The findings that (1) the effect of lysine binding is mimicked by G protein activators, including cholera toxin and GppNHp, and by PKC activators such as diacylglycerol, and (2) the facilitation by lysine is blocked by the G protein inhibitor, GDP-β-S, while facilitation by the direct PKC activator, diacylglycerol, is not inhibited, suggested that the facilitation by lysine is obligatorily transduced by a G protein, and that the action of diacylglycerol and PKC occur downstream from the GDP-β-S inhibitable G protein (Baxter and Morse, 1987). These observations thus indicated that the lysine receptor, G protein, and PKC act in a sequential cascade, possibly involving the G protein-dependent generation of the PKC-specific second messenger, diacylglycerol. Figure 5 schematically illustrates the suggested sequence of signal transduction events mediating the facilitation by lysine, and indicates the sites of activation and inhibition by the G protein effectors.

Cilia isolated from the larval epithelium contain sodium-independent receptors for lysine, and lysine binding to these receptors can be modulated by treatment of the cilia with G protein effectors. The sodium independence

Table I

Effect of G protein activators on lysine binding

	Treatment		
Parameter	Control	GppNHp	Cholera toxin
K_d (μ*M*)	.07 ± .05	1.2 ± .05	1.0 ± 0.6
B_{max} (fmol/μg)	9.3 ± 2.9	9.5 ± 0.4	10.0 ± 1

Radioligand binding analyses were performed with cilia, and K_d and B_{max} values determined for the high affinity binding site by Scatchard analysis of the equilibrium binding data as described. Binding studies were performed in the presence and absence of 0.1 m*M* GppNHp and with cilia pretreated with cholera toxin and NAD, as described in Materials and Methods.

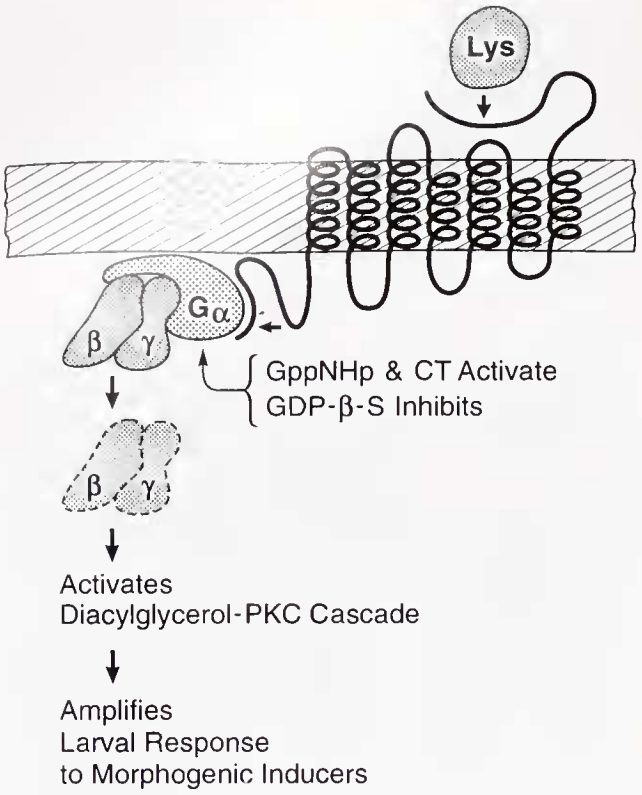

Figure 5. Schematic diagram of the hypothetical signal transduction pathway suggested to mediate the facilitation by lysine. Binding of lysine to an extracellular domain of the receptor protein (drawn here as a transmembrane protein) activates dissociation of the intracellular trimeric G protein. The released β and γ subunits of the G protein then activate the downstream cascade (apparently including a diacylglycerol-dependent activation of PKC) that results in the facilitation of larval settlement and metamorphosis. GppNHp and cholera toxin (CT) directly activate the G protein and mimic the facilitating effect of lysine. GDP-β-S inhibits the G protein and inhibits facilitation by lysine, but does not inhibit facilitation by diacylglycerol (*cf.* Baxter and Morse, 1987).

of the lysine receptors on the isolated cilia (Baxter, 1991) suggests that they may be true chemosensory receptors, as the binding of amino acids to transporters generally is sodium-dependent (Boge and Rigal, 1981; *cf.* Jaeckle and Manahan, 1989). Because the purified cilia are predominantly free of other cell structures, we can exclude the binding of lysine to synaptic receptors in this preparation.

Baxter (1991) first observed that cholera toxin-catalyzed labelling of the putative G protein α-subunit in the isolated cilia is stimulated significantly by the diamino acid receptor ligand lysine. This result, analogous to the direct increase in susceptibility to labelling caused by GppNHp (Fig. 4), suggests that the lysine receptor is functionally coupled to a cholera toxin-sensitive G protein in the purified cilia. Transmembrane receptors coupled to G proteins display a marked decrease in their affinity (increased K_d) for ligand in the presence of GTP analogs and other G protein activators (Stadel *et al.*, 1982). This effect is thought to result from the activation-induced dissociation of G protein from the receptor, reciprocally altering the receptor's binding affinity for ligand. Our observation of this effect on the ciliary lysine receptor in response to both GppNHp and cholera toxin (Table I), which interact with the ciliary G protein *in vitro* (Fig. 4) and can replace lysine as an amplifier of larval sensitivity to morphogen *in vivo* (Baxter and Morse, 1987), thus further supports the suggestion that the receptor is functionally coupled to a cholera toxin-sensitive G protein, and that the receptor and G protein reciprocally regulate one another. These results suggest that the lysine receptor on the larval cilia may be a member of the G-coupled transmembrane receptor superfamily (*cf.* Fig. 5).

G protein-dependent pathways of chemosensory signal transduction have been characterized in cilia from several vertebrates, including fish, amphibians, and mammals (Chen and Lancet, 1984; Pace *et al.*, 1985; Huque and Bruch, 1986; Pace and Lancet, 1986; Anholt *et al.*, 1987; Huque *et al.*, 1987; Jones and Reed, 1989). Other receptor-coupled G protein-dependent signal transduction cascades, similar to that involved in the lysine-stimulated amplifier pathway of the *Haliotis* larvae (Baxter and Morse, 1987), are common mediators of hormonal and other chemical signals controlling cell function and differentiation in a wide variety of systems, including mammals (Gilman, 1987; Freissmuth *et al.*, 1989; Bourne *et al.*, 1990).

Other similarities between larval and mammalian systems

Recently, cilia isolated from the *Haliotis* larvae have been found to contain mRNAs coding for two G protein α subunits. One is highly homologous to members of the G_q subfamily, while the other is closely related to the G_i/G_o group (Wodicka and Morse, 1991). It is striking that in the region of the deduced protein sequence corresponding to the two guanosine nucleotide binding domains, 50 of the 51 amino acid residues of the larval $G_q\alpha$ are identical to those in the protein from mammalian brain.

Other strong similarities have been found between receptor–transducer systems controlling neuronal activity and cellular differentiation in mammals and those controlling the settlement and metamorphosis of marine invertebrate larvae (Morse, 1985, 1990). The inducer recognized by the *Haliotis* larvae is a GABA-mimetic peptide produced by the algae on which the larvae are induced to settle (Morse and Morse, 1984). This peptide also binds strongly and specifically to GABA receptors purified from mammalian brain (H. Trapido-Rosenthal and A. N. C. Morse, in prep.). Similarly, we have found that the inducer of settlement and metamorphosis recognized by larvae of

the coral, *Agaricia humilis,* is a sulfated glycosaminoglycan that also is mitogenic for mammalian lymphocytes (Morse and Morse, 1991). Thus: (1) for larvae from two invertebrate phyla, the natural inducers of settlement and metamorphosis cross-react with mammalian receptors controlling cell proliferation, differentiation or function; and (2) the larval receptors and transducers thus far characterized in these pathways are functionally and structurally related to those in mammals. These findings suggest (beyond the probable evolutionary relatedness of the larval and mammalian systems) that characterization of the molecules controlling larval settlement and metamorphosis may point to the design of new regulators of mammalian cell function with usefulness in biotechnology and medicine.

Acknowledgments

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